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Cutting Edge: Histone Acetylation and Recombination at the TCRγ Locus Follows IL-7 Induction

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IL-7 signaling is required for V(D)J recombination at the TCRγ locus. We have recently reported that IL-7 controls chromatin accessibility for RAG-mediated cleavage. Inhibition of histone deacetylase substituted for the IL-7 signal, indicating a role for histone acetylation in altering chromatin accessibility. We found a greatly reduced histone 3 and histone 4 acetylation level in IL-7R−/− thymocytes in comparison with RAG−/− thymocytes or fetal thymocytes. Sterile transcripts, indicating an open chromatin configuration, were suppressed in IL-7R−/− and IL-7−/−RAG−/− thymocytes. Moreover, exogenously added IL-7 induced sterile transcripts from the TCRγ constant region in cultured thymocytes from IL-7−/− mice. This induction correlated with increased histone acetylation at the J-promoter and C-enhancer regulatory elements at the TCRγ locus. These results suggest that IL-7 regulates chromatin accessibility for V(D)J recombination by specifically altering histone acetylation within the TCRγ locus. The Journal of Immunology, 2001, 167: 6073–6077.

Variable diversity joining recombination is a site-specific recombination process that leads to the creation of a novel immune receptor gene repertoire (1). Recombination is crucial for lymphoid development, and aberrant regulation of this process may lead to immune deficiency or promote tumorigenesis. Recombination is initiated by the recombinase-activating gene products RAG-1 and RAG-2. The RAG dimer recognizes and specifically cleaves recombination signal sequences that flank immune receptor gene segments. The recombination process is tightly regulated in a stage- and lineage-specific manner. This level of control cannot be explained simply by the presence or absence of the RAG recombinase. Thus it had been postulated that access of the V(D)J recombinase to its target sequence is regulated and that chromatin modifications similar to those required for transcriptional processes participate in this regulation (2, 3). In support of this hypothesis, it was reported that transcription of unrearranged gene segments usually precedes V(D)J recombination and the absence of transcriptional regulatory sequences within the TCR locus results in inhibition of recombination. Thus, an open chromatin structure may allow access of the transcriptional machinery as well as access for the V(D)J recombinase. Recently it was reported that targeted deletion of regulatory sequences of the TCRγ and β locus that abrogate recombination correlates with the level of histone acetylation (4, 5). This suggests another close relationship between the regulation of chromatin accessibility for transcription as well as the process of V(D)J recombination.

IL-7 is required for normal lymphoid development and specifically for V(D)J recombination at the TCRγ locus (reviewed in Refs. 6–8). Deletions of different components of the IL-7 signal transduction pathway inhibit γδ T cell development and leads to a specific suppression of V(D)J recombination at the TCRγ locus (9–12). Although IL-7 provides survival functions for pro-T cells, the defect in recombination cannot be corrected by substituting the IL-7 signal with a survival signal. Thus a bcl-2 transgene did not restore V(D)J recombination at the TCRγ locus in IL-7R−/− mice (12). We have previously reported that in the absence of the IL-7Rα signal, T cell precursors fail to initiate cleavage at the TCRγ locus and that this failure is due to an inaccessible state of chromatin for RAG-mediated cleavage (13, 14). The need for the IL-7Rα signal can be replaced by Trichostatin A, a specific inhibitor of histone deacetylases, suggesting a role of histone acetylation in the opening of chromatin structure induced by IL-7. However, Trichostatin A treatment induces a global, rather than locus-specific, acetylation of histones, and thus its mechanism of mimicking the IL-7 effect on the TCRγ locus might have been indirect. Thus, in the present study we address the question of how IL-7 signaling modifies chromatin to allow access for the V(D)J recombinase. We examined whether IL-7 induces specifically histone acetylation directly at regulatory sites within the TCRγ locus.

Materials and Methods

Mice

Embryonic thymus were derived from day 15 gestation of C57BL/6 mice (Animal Production, Frederick, MD). IL-7R−/− mice and Rag-2−/− mice (The Jackson Laboratory, Bar Harbor, ME) were 4–8 wk of age. The generation of IL-7−/−Rag-1−/− were kindly provided by R. Murray (EOS Biotechnology, San Francisco, CA) (9). Animal care was provided in accordance with the procedures outlined in the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health Publication No. 85-23, 1985).

Primers and probes

The locations of primers and probes within the TCRγ locus are shown in Fig. 1. The primers for detection of gene rearrangement and sterile transcripts were derived from sequences as previously published (13, 14).
following oligonucleotides were used in the chromatin immunoprecipitation (ChIP)-PCR assay: 1) upstream: S5'-GGCGAAGAGAAGACAACTG-3'; S5'-GGGACAGTCTATTTAGTC-3'; and probe: S5'-GGTCTGATGCTACTTCTTCC-3'. 2) VβHs: S5'-GCCTCCTCTCCC TTCTTG-3'; S5'-TGTGGCAGACATCCTGAGTG-3'; and S5'-GGGAA CACCAAGGCGGACG-3'. 3) Jy promoter: S5'-CCCTTCTCGAGAA TATATCCC-3'; AS5'-TAATTTCCGAGAATCCTGTG-3'; and P5'-ACATTTGCTTGGTGAGAC-3'. 4) Constant region enhancer: S5'-GTGGCTCTCCGAGAATGTTAAAG-3'; S5'-CTAAATGAGCTTGTGAAATG-3' and P5'-AGGAGGATCAGGAGGGCTCTG-3'. 5) Downstream: S5'-AGGCCTTGCAAGAGAAGACG-3'; S5'-TAGAAC CAGAGGTCTCCG-3'; and P5'-CAGAGGACTCCGAGGCTAGGG-3'. 6) δ Enhancer: S5'-CAAAGTATACGCCCCGACCCCA-3'; AS5'-CACCA AACTTGAATAACCC-3'; and P5'-AAAGAGATAGCAGGGCTCTG-3'. 7) Oct-2: S5'-TGGAGAGGCTTCTAGGTTG-3'; S5'-GAGGCTTCTGGATCTGTGG-3'; and P5'-ATCAACGCTGCTCAACAGG-3'. 8) Pγ2-5': S5'-TACGAATGCGAGGACGCTTGG-3'; AS5'-GAGCAATGCTCGTGGAAAGG-3'; and P5'-GGGAGGAGCACGAAAGG-3'.

All these primer sets were designed to give a PCR product size ranging from 110 to 160 bp.

**PCR and RT-PCR analysis**

The PCR after ChIP was performed with Supermix (Life Technologies, manufacturer) according to the manufacturer's protocol (acetyl-histone H3 or H4 ChIP assay kit; Upstate manufacturer). Hybridization was performed in Rapid-hyb buffer (Schleicher & Schuell, Keene, NH). Hybridization was performed in Rapid-hyb buffer (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's protocol and visualized by autoradiography.

**ChIPs assay**

The procedure of chromatin immunoprecipitation was modified from the manufacturer's protocol (acetyl-histone H3 or H4 ChIP assay kit; Upstate Biotechnology, Lake Placid, NY) as follows. Thymocytes were cross-linked with 1% formaldehyde for 12 min at 37°C and the reaction was stopped by adding glycine to a final concentration of 125 mM for 5 min. The cells were washed twice in ice-cold PBS and lysed in SDS lysis buffer. Sonication was performed on ice and the samples were cooled on dry ice between the pulses, but care was taken not to freeze the samples. The length of the DNA fragments averaged between 200 and 500 bp. After precleaving, 10% of each sample was saved as input fraction. Immunoprecipitation was performed using specific Abs against acetylated histone 3 (5 µg/ml) and histone 4 (4 µg/ml), or normal rabbit IgG (5 µg/ml) as control. After incubation for 16 h at 4°C with agitation, the Ab/histone/DNA complex was captured by using salmon sperm DNA/protein A-agarose slurry for 1 h rotating at 4°C. The supernatant of each sample was kept as unbound fraction. The complex was eluted and the cross-linking was reversed. After purification, the DNA concentration was determined and diluted in three-fold series (20 ng, 67 ng, and 2.33 ng) for PCR amplification. The blot result was imaged by PhosphorImager and quantitated by ImageQuant v5.0 software (Molecular Dynamics, Sunnyvale, CA). The relative acetylated enrichment as shown in the figures was determined from

the ratio of bound/input fractions that was very similar to the ratio formed by bound/unbound control fractions. The results shown in the figures are expressed as means ± SE from three to five independent ChIP experiments.

**Results and Discussion**

**Hyperacetylation of histones at the TCRγ locus in normal fetal thymocytes**

To determine whether IL-7 stimulation effects histone acetylation at the TCRγ locus, we first determined the status of acetylation in the presence of IL-7 during normal development. TCRγ locus rearrangement occurs in thymocytes between days 14 and 16 of murine gestation. During this period, sterile transcripts and recombination intermediates are detected, indicating an open chromatin configuration and an actively occurring recombination process, respectively (14). Thus, fetal thymocytes were used as a nuclear source for ChIP using specific Abs against acetylated histones. The acetylation of N-terminal lysine residues of histone 3 as well as histone 4, components of the mononucleosomes, are thought to result in an open chromatin structure (2, 3, 15). Fragmentation of chromatin after cross-linking allowed for specific analysis of small regulatory sequences (average size 200–500 bp). The murine TCRγ locus extends 40–50 kbp comprising four variable region gene segments and one joining and constant region segment (Fig. 1). We first investigated the status of histone 3 and histone 4 acetylation at regulatory sites recently identified as transcriptional response elements within the TCRγ locus: the DNase I hypersensitive site A (V-HsA), an enhancer-like element between the Vδ and V2 genes, the J region promoter (J-promoter), or the constant region enhancer (C-enhancer) (12, 16, 17). In addition, the enhancer of the TCRγ locus (Eb) was examined as a positive control. A representative ChIP experiment (Fig. 2A) indicates specific acetylation at histone 3 as well as histone 4 for all three regulatory sites in comparison with the control immunoprecipitation. A critical question is whether this demonstrated acetylation level found at the TCRγ locus is specifically elevated in comparison with other sites in the genome. To evaluate this question, the ratio was calculated between the specifically precipitated material and the input fraction and expressed as fold acetylation, as shown in Fig. 2B, summarizing five experiments. This ratio indicates how much a specific sequence is enriched in the precipitated fraction in comparison with the whole genome (Fig. 2B). Thus the J-promoter, the C-enhancer, and the HsA sites are 22-, 10-, and 13-fold enriched in the acetylated H3 fraction and 35-, 15-, and 25-fold in the acetylated H4 fraction. This suggests a locus-specific enhancement of histone acetylation at the TCRγ locus. In contrast, the tissue-specific expressed genes Oct-2 (B cell-specific) and Pγ2-5 (testis-specific) are not enriched in the precipitated fraction, indicating no significant enhancement for acetylation in comparison with the overall genome. To determine the borders of hyperacetylation at the TCRγ locus, we analyzed additional sequences that lack any known regulatory function. Whereas the upstream site (1300 bp upstream of Vγ5) still showed a 4-fold enrichment (2-fold over Oct-2), the downstream sequence (1700 bp downstream of the C-enhancer) was indistinguishable in its H3 or H4 acetylation level from the control gene Oct-2. Thus the three known regulatory sites of the TCRγ locus are hyperacetylated in comparison with the global genome and at least two other tissue-specific genes. Thus the acetylation is rather focused within the locus, tapering off with increasing distance from the regulatory sites.

**Reduced histone acetylation at the TCRγ locus in the absence of IL-7Rα signaling**

Next we addressed the question of whether hyperacetylation as observed at the TCRγ locus during normal T cell development...
occurs in response to IL-7Rα signaling. Recently we reported a defect in V(D)J recombination and a reduced chromatin accessibility in IL-7Rα-/- thymocytes compared with those with RAG-/- thymocytes. Deletion of RAG-1 or RAG-2 genes leads to an abrogation of V(D)J recombination and an early arrest of T cell development at a stage similar than that of day 15 fetal thymocytes. However, RAG-/- thymocytes have detectable sterile transcripts and their chromatin shows normal accessibility to RAG-mediated cleavage in vitro in contrast to IL-7Rα-/- thymocytes (14). Thus we compared the level of histone acetylation at the TCRγ locus between IL-7Rα-/- thymocytes and RAG-/- thymocytes. As shown in Fig. 3A, the level of histone acetylation was greatly reduced in the absence of IL-7Rα signaling. The enrichment for acetylation in RAG-/- thymocytes was 20-, 26-, and 19-fold at J-promoter, C-enhancer, and V-HsA sites for H3 and 22-, 21-, and 16-fold for H4, and thus comparable with the hyperacetylation in fetal thymocytes (Fig. 3B). The slight differences between RAG-/- and fetal thymocytes possibly reflect distinct developmental stages (the pro-T3 stage for RAG-/- thymocytes in contrast with the pro-T1/T2 stage for d14 fetal thymocytes). In both controls all three regulatory sites were markedly hyperacetylated. In contrast, the chromatin derived from IL-7Rα-/- thymocytes showed an overall histone acetylation level indistinguishable from the control site within the Oct-2 gene. The reduction in acetylation was 12- to 22-fold for H3 and 6- to 10-fold for H4 comparing IL-7Rα-/- with RAG-/- acetylation levels. Thus the specific histone acetylation at the regulatory sites within the TCRγ locus that is observed during normal development is dependent on IL-7Rα signaling.

**Induction of sterile transcripts in IL-7-/-RAG-/- thymocytes by IL-7**

IL-7 is also an important survival signal for T cell precursors, although Bcl-2 cannot rescue the defective TCRγ rearrangement in IL-7Rα defective mice (12). To determine whether IL-7 can directly affect accessibility at the TCRγ locus instead of promoting accessibility indirectly through enhanced viability, we examined the induction of sterile transcripts. Transcription of unarranged gene segments usually precedes V(D)J recombination and is considered an indicator of accessible chromatin. Previously we have reported that sterile transcripts are reduced in IL-7Rα-/- thymocytes in contrast to RAG-/- thymocytes (13) (Fig. 4A). We also detected a suppression of sterile transcripts for Vγ2 and Vγ3 and the constant region in IL-7-/- RAG-/- thymocytes, indicating that at least part of the detrimental effect of IL-7Rα deletion is due to IL-7 itself (rather than, for example, TSLP, which also binds IL-7Rα). Recently it had been reported that IL-7 can induce TCRγ transcripts within 5 h in CD4+ T cells purified from adult thymus (18). Similarly, we were able to induce constant region transcripts in IL-7-/- RAG-/- thymocytes within 5 h of stimulation with IL-7 in culture (Fig. 4A). This rapid process suggests that IL-7 signaling is directly able to induce opening of chromatin in thymic precursors, rather than promoting cell survival or growth, indirectly affecting chromatin. We could not observe Vγ2 and Vγ3 transcripts in this time period, although the reduced acetylation levels at the HsA site would suggest a control by IL-7Rα signaling as well. However, we observed reduced viability of IL-7-/- RAG-/- thymocytes over time in culture that was not restored by addition of additional cytokines such as stem cell factor. It remains possible that a different ligand of IL-7Rα, such as TSLP (19), could provide long-term survival in culture or that TSLP may specifically participate in the control of Vγ2 and Vγ3 transcription. Recently it was reported that the histone acetylation levels at the recombination recognition sites at Vγ2 and Vγ3 are high in fetal thymocytes, as reported in this work, but distinctly regulated in adult thymocytes (being lower at Vγ3) (20). This suggests regulatory factors other than simply IL-7 alone being involved in the control of a
Histone acetylation at the TCRγ locus is induced in IL-7−/− RAG−/− thymocytes by IL-7

The induction of constant region transcripts by IL-7 within 5 h supports the idea that IL-7 directly effects chromatin accessibility at the TCRγ locus. To determine whether histone acetylation contributes to the inducible opening of chromatin at the TCRγ locus by IL-7 and to determine which regulatory sites are involved in this induction, ChIP experiments were performed. Fig. 4B summarizes a quantitative analysis of three independent ChIP experiments using thymocytes derived from IL-7−/− RAG−/− mice and cultured with IL-7 in vitro for 5 h or left untreated. The level of H3 or H4 acetylation at the J-promoter or C-enhancer in untreated IL-7−/− RAG−/− thymocytes was undistinguishable from the level observed at the Oct-2 gene. This underacetylation corresponds with the low acetylation level detected in IL-7Rα−/− thymocytes. Within 5 h of IL-7 stimulation histone acetylation levels changed at the J-promoter and C-enhancer. Although the histone acetylation after stimulation with IL-7 was much smaller than that observed in wild-type thymocytes (see Fig. 2) the induction by IL-7 was three- to four-fold for H3 and H4 above the untreated control (Fig. 4B). The J-promoter and C-enhancer are thought to drive sterile constant region transcripts. Thus the induction of histone acetylation levels correlated closely with the induction of sterile transcripts. These results suggest that the IL-7 signal transduction pathway controls TCRγ locus recombination by increasing acetylation of histones at specific cis-acting elements.

The precise signal transduction pathway leading from the IL-7R to the site of specific histone acetylation within the TCRγ locus is not yet known, although the transcription factor Stat5 would be a candidate. IL-7 can activate Stat5, and there is strong evidence that Stat5 can induce histone acetylation. Moreover, Stat5 can bind to sequences within the J-promoter (the C-enhancer and V-HsA element contain potential binding sites) (12). The most compelling evidence that Stat5 could mediate the observed acetylation effect comes from the report that a transgene expressing Stat5 restores TCRγ recombination in IL-7Rα deleted lymphoid precursor cells (12). Stat5 has been demonstrated to associate with the interacting protein Nmi and with the histone acetylases CBP/P300 (21). Thus activation of Stat5 by IL-7 could lead to site-specific recruitment of histone acetylases at the TCRγ locus. In contrast, knockout of Stat5a/b was not reported to be deficient in γδ T cells, implicating additional mediators.

How can site-specific histone acetylation regulate V(D)J recombination? Previous work suggested that mononucleosomes could inhibit RAG-mediated cleavage in vitro (22, 23). This inhibition is thought to be caused by inhibiting access of the RAG dimer to the recombination signal sequence as well as to reducing cleavage capacity of the RAG recombinase (23). How can histone acetylation overcome this suppression? Acetylation of histone tails may affect the structure of the mononucleosome or participate in unfolding of higher chromatin structure. Acetylated histone tails may also serve as flags for recruitment of other chromatin remodeling complexes. ATP-dependent complexes such as SWI/SNF complexes may disrupt chromatin structures by sliding mononucleosomes along the DNA, thus providing better access for the RAG recombinase. In support of this model, it was recently reported that both acetylation and SWI/SNF-dependent chromatin remodeling act in concert to promote RAG-mediated cleavage in vitro (23). However, it is not yet known whether SWI/SNF-dependent remodeling contributes in vivo to accessibility for V(D)J recombination or whether other recombination-specific chromatin remodeling activities exist. The use of IL-7 to induce specifically TCRγ rearrangement may serve as a valuable in vivo model to study the molecular mechanism for control of chromatin accessibility for V(D)J recombination.

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References


